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PRINCIPAL INVESTIGATOR: Kathy J. Helzlsouer, M.D., M.H.S.

CONTRACTING ORGANIZATION: Johns Hopkins University  
Baltimore, Maryland 21205

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University Baltimore, Maryland 21205  E-MAIL: khelzls@phnet.sph.jhu.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
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*Kathy J. Hefner*  
PI - Signature

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Date

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## **1. INTRODUCTION**

The purpose of the study is to assess whether suboptimal repair of DNA damage is associated with increased breast cancer risk, to assess the possible interaction between DNA repair proficiency and ionizing radiation exposure, and to evaluate the inheritance pattern of suboptimal DNA repair proficiency.

## **2. BODY: ANNUAL SUMMARY YEAR 2**

### **Case-control and Family studies, Tasks 1-6**

#### **Task 1**

We developed study brochures and finalized questionnaires in year 1.

#### **Task 2**

To date, we have recruited 76 women to the case-control study, and are adding recruitment days and clinics, and will recruit 3 to 5 days per week over the next year. Accrual to the case-control study, 300 cases and controls, should be reached by the end of April. Because of difficulty with the assay, family study recruitment could not be done. We will begin recruitment of family members to the family study, 150 family members from 30-40 families, during year 3.

#### **Task 3**

We are collecting questionnaire data and blood samples as we recruit participants to the study.

#### **Task 4**

### **Performing DNA repair assays: Assay Development in Year 1**

We transferred the DNA repair proficiency assay developed at NIH by Sanford and Parshad (1) and used in our pilot study in 1993-5, to our laboratory in 1997. Transferring the assay required adapting the assay from an ionizing radiation source to a gamma (cesium chloride) radiation source, because an ionizing source was not available. At NIH, lymphocytes were stimulated with PHA in 20 ml of media on day one, and incubated for 72 hours before irradiation with 58 cGy ionizing radiation on day four. Lymphocytes were allowed to repair at 37°C for 0.5 hour without colcemid, and then for one hour with colcemid, to arrest cells in metaphase. Repaired cells were lysed at 37°C, and fixed at 4°C, and three slides were made for each sample assayed. Slides were examined to identify fifty metaphase (dividing) cells, and metaphase cells were examined for chromatid damage (breaks and gaps). Chromatid breaks show a discontinuity with displacement of the broken segment. Chromatid gaps show a discontinuity with no displacement, and were scored only if the discontinuity was longer than the chromatid width.

To assess the reliability of the DNA repair assay in our laboratory we used blood from a donor who had good repair eleven times in our pilot study at NIH. During the early part of assay development, irradiating lymphocytes from this quality control standard for good repair with 50 cGy gamma radiation resulted in fewer metaphase cells than irradiating with 25 cGy, indicating that 50 cGy may be blocking mitosis or killing cells. Therefore we decided to use 25 cGy gamma radiation in our assay, and found that adding colcemid at 0.5 hour for one hour, was the best time for repair with this radiation dose. Under these conditions, lymphocytes from our quality control standard for good repair, and from a donor who had poor repair in our pilot study at NIH, had repair results consistent with NIH results.

Initially repeatability of our assay was very good. We assayed donors' lymphocytes in triplicate, using 25 cGy radiation and adding colcemid at 0.5 hour for one hour, in two assays in May and June of 1998 (Table 1). Our quality control standard for good repair had 18, 19, and 16 breaks and gaps per fifty metaphase cells in the first assay; and 14, 15, and 16 breaks and gaps per fifty metaphase cells in the second assay. These numbers indicate good repair in our laboratory in this donor who always had good repair at NIH. We also assayed cells from a new donor, never assayed at NIH, and from a donor who had poor repair at NIH. The new donor had 16, 18, and 18 breaks and gaps per fifty metaphase cells in our assay, indicating good repair in our new donor. The donor with poor repair at NIH had 35, 30, and 34 breaks and gaps per fifty metaphase cells in our assay, about two times the breaks and gaps we found in our quality control standard for good repair, and in our new donor.

**Table 1. Repeatability: Chromatid breaks and gaps and repair status, after 25 cGy gamma irradiation and 0.5 hour of repair time before adding colcemid for one hour**

Date of assay	Donor	Breaks and gaps per 50 metaphase cells	Repair status	
			Pilot study at NIH	Our laboratory
5/11/98	STD GR <sup>1</sup>	18, 19, 16	Good	Good
	New Donor 101 <sup>2</sup>	16, 18, 18	Not done	Good
6/1/98	STD GR <sup>1</sup>	14, 15, 16	Good	Good
	NIH PR 104 <sup>3</sup>	35, 30, 34	Poor	Poor

<sup>1</sup> Donor had good repair multiple time in our pilot study at NIH and is our quality control standard for good repair.

<sup>2</sup> Donor's blood was never assayed at NIH.

<sup>3</sup> Donor had poor repair at NIH.

Because of good results in our repeatability assays, we started recruiting patients to the case-control study in June of 1998. We included our quality control standard for good repair in some of our assays, and found that she had good repair in our June assay, but poor repair in an assay we did in July. We stopped recruiting patients to determine the reason for inconsistent results. In one of two assays we did in August of 1998, this standard had poor repair again. In September of 1998, this standard and our new donor who had good repair in our repeatability assays in May and June of 1998, both had good and poor repair.

## Performing DNA repair assays: Assay Development in Year 2

The inconsistent results in our assays in year 1 made us review all aspects of the assay in year 2, and we have attempted to optimize conditions in the assay. Possible conditions leading to inconsistent results include changes in temperature and pH of the media before and during repair time, and bacterial contamination (2).

By the start of year 2 we had optimized temperature control throughout our assay. We transported cells in a 37°C Styrofoam water bath, to the Oncology Center, where a 37°C warm room and irradiator are located in close proximity. We started using the warm room in September of 1998, to warm cells after transport and during repair time. After all repair time, we chilled cells in ice-water bath to stop repair, and returned the cells to our laboratory for lysing and fixing.

In November of 1998, we compared growing and assaying cells from our quality control standard for good repair, in 5% and 10% CO<sub>2</sub>. Cells grown in 10% CO<sub>2</sub> were gassed with 10% CO<sub>2</sub> in air, in a portable tissue culture chamber which we sealed after gassing, and placed in our 5% CO<sub>2</sub> incubator. After irradiating cells, we added fresh media pre-equilibrated with 5% CO<sub>2</sub> to our cells grown in 5% CO<sub>2</sub>, and fresh media pre-equilibrated with 10% CO<sub>2</sub> to our cells grown in 10% CO<sub>2</sub>. Cells from our quality control standard for good repair had poor repair (28 and 31 breaks and gaps per 50 metaphase cells) under 5% CO<sub>2</sub> conditions, but good

repair (15 and 16 breaks and gaps per 50 metaphase cells), under 10% CO<sub>2</sub> conditions. As a result we switched to growing and assaying cells in 10% CO<sub>2</sub>.

In a second assay in November of 1998, we grew cells from three donors, in the portable tissue culture chamber gassed with 10% CO<sub>2</sub>. Our new donor who had good repair in our repeatability assays in May and June of 1998 had good repair in this assay, but our quality control standard for good repair had poor repair, indicating that we still had problems with our assay after switching to 10% CO<sub>2</sub>. The parent of a child diagnosed with ataxia telangiectasia had poor repair in this assay, as expected.

In January through February of 1999, we started growing cells directly in a 10% CO<sub>2</sub> incubator, instead of using the portable chamber gassed with 10% CO<sub>2</sub>. We did three assays from cells grown in the 10% incubator, irradiating cells with 25 cGy radiation and letting cells repair for 0.5 hour before adding colcemid for one hour. Results were inconsistent (Table 2), and overall the metaphase cell preparations were of poor quality or contaminated. In the January assay, metaphase cells were few in number and chromosomes were poorly spread. In the first of the February assays, three of six sets of slides (from two donors assayed in triplicate) were contaminated, and there were too few metaphase cells in a fourth set to count breaks and gaps. In the second February assay, chromosomes were poorly spread. We suspect that the inconsistent results in these assays may be due to bacterial contamination from the incubator, since bacterial contamination can cause poor chromosome spreads and few metaphase cells. Bacterial contamination can cause erratic results in the assay, by enhancing the susceptibility of cells to radiation damage, hindering repair processes, protecting against damage, or by facilitating repair (2). We stopped growing cells in the 10% CO<sub>2</sub> incubator, and resumed growing cells in our portable chamber gassed with 10% CO<sub>2</sub>.

**Table 2. Chromatid breaks and gaps and repair status, after 25 cGy gamma radiation and 0.5 hour of repair time before adding colcemid for one hour, in cells that may have been contaminated**

Date of assay	Donor	Breaks and gaps per 50 metaphase cells	Repair status	
			Pilot study at NIH	Our laboratory
1/29/99	STD GR <sup>1</sup>	15, 32	Good	Good + Poor
	New Donor 101 <sup>2</sup>	15, 31	NA	Good + Poor
	AT Parent <sup>3</sup>	13, 14	NA <sup>3</sup>	Good
2/5/99	STD GR <sup>1</sup>	31	Good	Poor
	NIH PR 107 <sup>4</sup>	13	Poor	Good
2/22/99	STD GR <sup>1</sup>	30, 34, 14	Good	Good + Poor

<sup>1</sup> Donor is our quality control standard for good repair.

<sup>2</sup> Donor's blood was never assayed at NIH, but donor had good repair in our repeatability assays (Table 1).

<sup>3</sup> Donor is a parent of a child diagnosed as having ataxia telangiectasia and is expected to have poor repair in our assay.

<sup>4</sup> Donor had poor repair at NIH.

In March of 1999 we resumed growing cells in the portable tissue culture chamber gassed with 10% CO<sub>2</sub>, and let cells repair for 0.5, 0.75, and 1 hour before adding colcemid, to see if the longer repair times would improve repeatability. Repeatability was good at all time points in this assay; breaks and gaps in our quality control standard for good repair were 20 and 15 at 0.5 hour, 19 and 17 at 0.75 hour, and 22 and 15 at 1 hour.

In April of 1999, we incorporated modifications that Scott et al. made to decrease experimental variability in this assay (3,4). Because centrifuging cells before irradiating can slow progression of cells into

metaphase (3), Scott pipetted 15 ml of supernate from above cells at 71 hours, replaced this with fresh media pre-equilibrated with 5% CO<sub>2</sub>, and then irradiated cells one hour later. Scott also lysed cells at 4°C, instead of at ambient temperature, because repair may continue to varying extents at ambient temperature (3). We incorporated Scott's modifications in two assays we did in April and May of 1999, except that we continued to grow cells in 10% CO<sub>2</sub> in our portable tissue culture chamber (Table 3).

**Table 3. Chromatid breaks and gaps and repair status, after 25 cGy gamma radiation and 0.5 hour of repair time before adding colcemid for one hour, with and without Scott's modifications**

Date of assay	Breaks and gaps per 50 metaphase cells		Repair Status	
	with Scott's modifications	w/o Scott's modifications	with Scott's modifications	w/o Scott's modifications
4/23/99 <sup>1</sup>	16, 43	14, 46	Good + Poor	Good + Poor
5/10/99 <sup>1</sup>	16, 20	Not done	16, 20	Not applicable

<sup>1</sup> Cells are from our quality control standard for good repair.

Continuing to use Scott's modifications, in May of 1999 we irradiated cells from our quality control standard for good repair, in three flasks, then split cells from each flask into two tubes, and immediately added colcemid, for only 30 minutes, to minimize repair time. There were 57, 56, 56, 59, 54, and 55 breaks and gaps per 50 metaphase cells, indicating excellent repeatability when cells were only allowed to repair long enough to collect cells in metaphase.

In June through July of 1999 we let cells repair for three hours, the time allowed for DNA repair in another laboratory at our institution, before adding colcemid for one hour (Table 4). We continued to use Scott's modifications, but grew cells in media directly pre-equilibrated with 10% CO<sub>2</sub>, instead of using the portable chamber, and irradiated cells at 25 cGy in June, and at 25 cGy and 100 cGy in July. In June, cells from our quality control standard for good repair had no breaks or gaps when colcemid was added at three hours after irradiation; in July, this standard had 6 breaks and gaps to our NIH poor repairer's 14 breaks and gaps, when cells were irradiated with 100 cGy radiation and allowed to repair for three hours before colcemid was added.

**Table 4. Chromatid breaks and gaps after 25 and 100 cGy gamma radiation and three hours of repair time before adding colcemid for one hour**

Assay	Donor	Radiation Dose	Breaks + Gaps per 50 metaphase cells
6/18/99	STD GR <sup>1</sup>	25	0
7/23/99	STD GR <sup>1</sup>	25	4
		100	6
	NIH PR 107 <sup>2</sup>	25	7
		100	14

<sup>1</sup> Donor is our quality control standard for good repair.

<sup>2</sup> Donor had poor repair at NIH.

In August of 1999 we cut repair time back from three to two hours before adding colcemid, to try to get better discrimination between good and poor repair. We irradiated cells from our quality control standard for good repair and a from a donor with poor repair at NIH, with 100 cGy radiation, and allowed repair for two hours before adding colcemid. Both donors had few metaphase cells, and similar breaks and gaps, 10 and 11 per 50 metaphase cells. All chromosomes were poorly spread, and second sets of slides from these samples assayed



in duplicate had too few cells to count breaks and gaps, indicating that cells may have been contaminated.

In September we irradiated cells at 50 cGy, the radiation dose that Scott used, and compared letting cells repair for 0.5 hour and for one hour, before adding colcemid for one hour (Table 5). We let cells repair in a 37°C water bath in our laboratory, because the warm room might have been a source of bacterial contamination. Our donor with poor repair at NIH had 1.4 times as many breaks and gaps as our quality control standard for good repair, at the 0.5 hour time point; and 2 times as many breaks and gaps at the one hour time point. These results were the most promising and consistent. We have now resumed the assay, using the 37°C water bath and adding colcemid at one hour after irradiating cells, instead of at 0.5 hour after irradiating cells. We will continue to closely monitor results, with inclusion of a quality control sample with each run. Samples collected previously, while we were developing the assay, were collected with the intention of looking at polymorphisms and DNA repair genes.

**Table 5. Chromatid breaks and gaps after 50 cGy gamma radiation, and 0.5 and 1 hour of repair time in a 37°C water bath, before adding colcemid for one hour**

Assay	Donor	Repair time before adding colcemid	Breaks and gaps per 50 metaphase cells
9/13/99	STD GR <sup>1</sup>	0.5 H	26
		1 H	11
	NIH PR 108 <sup>2</sup>	0.5 H	37
		1 H	23

<sup>1</sup> Donor is our quality control standard for good repair.

<sup>2</sup> Donor had poor repair at NIH.

The modifications we have incorporated in our assay in year 2 to optimize conditions for repeatability in the DNA repair assay are as follows:

1. We grow cells in 10% CO<sub>2</sub> in air, instead of in 5% CO<sub>2</sub>, to better maintain a pH near 7.
2. We stopped centrifuging cells before irradiating.
3. We irradiate cells with 50 cGy irradiation instead of 25 cGy irradiation.
4. We let cells repair in 37°C water bath in our laboratory, instead of borrowing a warm room for repair time, to reduce the risk of bacterial contamination during repair time.
5. We add colcemid after one hour of repair time, instead of after 0.5 hour of repair time.
6. We lyse cells at 4°C, instead of at ambient temperature, and modified how we lyse and fix cells, to minimize loss of metaphase cells.

#### **Task 5**

Entry of questionnaire data, family relationships, and DNA repair results is ongoing.

#### **Task 6**

Data analysis and writing of the report are pending completion of Tasks 1-5.

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